The Number of Alveoli in the Human Lung

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The number of alveoli is a key structural determinant of lung architecture. A design-based stereologic approach was used for the direct and unbiased estimation of alveolar number in the human lung. The principle is based on two-dimensional topology in three-dimensional space and is free of assumptions on the shape, size, or spatial orientation of alveoli. Alveolar number is estimated by counting their openings at the level of the free septal edges, where they form a two-dimensional network. Mathematically, the Euler number of this network is estimated using physical disectors at a light microscopic level. In six adult human lungs, the mean alveolar number was 480 million (range: 274–790 million; coefficient of variation: 37%). Alveolar number was closely related to total lung volume, with larger lungs having considerably more alveoli. The mean size of a single alveolus was rather constant with $4.2 \times 10^6 \mu m^3$ (range: $3.3–4.8 \times 10^6 \mu m^3$; coefficient of variation: 10%), irrespective of the lung size. One cubic millimeter lung parenchyma would then contain around 170 alveoli. The method proved to be very efficient and easy to apply in practice. Future applications will show this approach to be an important addition to design-based stereologic methods for the quantitative analysis of lung structure.

Keywords: connectivity; disector; Euler number; morphometry; stereology

The diffusing capacity of the lung is structurally limited by the size of the alveolar surface area and the thickness of the blood–gas barrier (1). To be efficient, a sufficiently large surface area for gas exchange must be crammed into the limited space available in the thoracic cavity. As a consequence, the lung's internal structure shows a gas exchange surface that is divided into a large number of small subunits connected to a branched conducting airway system (2). The smallest gas exchange unit within the respiratory parenchyma is the alveolus. The total number of alveoli in the lung is therefore one of the key structural determinants of the architecture of the respiratory parenchyma and, therefore, for adequate lung function.

Although the question "How many alveoli are there in the human lung?" (3–5) seems to be a very simple one, it is indeed difficult to answer in practice. This is largely due to the fact that alveoli are not discrete, separate particles but rather a set of open saccules or pronounced surface irregularities with openings making them impossible to define unambiguously in single histologic sections. Knowing the alveolar number, however, is of general importance for the understanding of lung development and disease.

The measurement of lung structure yields quantitative data for parameters like volume, surface area, length, cell number, and cell size. Whereas measurement of structure in general is known as morphometry, the methods to obtain these data in microscopy are referred to as stereologic methods (6). Stereology can be defined as the science of sampling structures with geometric probes (7). To generate counting events between the structures and the probes, the sum of the dimension of the parameter and the dimension of the probe has to be at least three, namely the dimension of the reference space (6, 7). Therefore, the number of objects in space can only be estimated without bias by using three-dimensional probes. The disector (8) is a three-dimensional stereologic probe. The disector principle can be considered pivotal in modern design-based or unbiased stereology (9–11). Although discrete three-dimensional particles may always be counted in disectors of sufficient resolution, the lack of a well-defined counting unit essentially makes it impossible to count alveoli in the ordinary way with disectors.

The aim of the present study was to establish a design-based stereologic approach for the direct and unbiased estimation of alveolar number (*see* Reference 12) in the human lung and to provide reference data for the normal human lung. As an extension of the disector method for particle number estimation, the method is based on a two-dimensional topology in a three-dimensional space and is free of assumptions on the shape, size, or spatial orientation of alveoli. Alveolar number is estimated by counting their openings, which are regarded at the level of the free septal edges, where they form a two-dimensional network in three-dimensional space. Mathematically, the Euler number of this network is estimated using physical disectors at a light microscopic level. The method is developed specifically for the estimation of alveolar number, although the concept of the Euler number is approximately 250 years old. Depending on the overall sampling strategy adopted, it is possible to estimate the total alveolar number and mean size using either a fractionator design (13) or a disector–Cavalieri combination, the latter of which is used in the present study. Because number estimation is completely independent of the orientation distribution, the sections may be sampled according to so-called vertical or isotropic uniform random designs or just with an arbitrary orientation distribution, making it effortless to combine the method with all other stereologic estimators of interest for lung quantitation.

METHODS

Fixation, Sampling, and Processing

In six cases of single lung transplantation, four females and two males, the contralateral human donor lung was used for microscopic analysis, provided it could not be matched to another suitable recipient by The Eurotransplant Foundation Centre, Leiden, The Netherlands (*see* Reference 14 and Table E1 in the online supplement). The lungs were fixed by airway instillation. The total volume of the lungs, V(lung), was determined by the Cavalieri principle. Systematic, uniformly random

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samples were then taken and processed as described in detail previously (15). The samples were osmicated, dehydrated, and embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer, Weinheim, Germany). In comparison with paraffin embedding, glycol methacrylate embedding offers a much lesser degree of tissue deformation and is therefore ideally suited for many stereologic studies (16, 17). Additional details on the fixation and sampling protocol as well as on the method for lung volume measurement are provided in an online supplement.

Stereologic Analysis

Stereologic analysis was performed with an Axioskop light microscope (Zeiss, Oberkochen, Germany) equipped with a computer-assisted stereology system (CAST 2.0; Olympus, Ballerup, Denmark). Four to five blocks from each lung were analyzed. From each block, two adjacent sections of 9 μm thickness were aligned in parallel on one glass slide. Because there was sufficient contrast due to osmication, the sections were analyzed unstained.

Using point counting on the first of the two adjacent sections with a 2.5 Plan-Neofluar (Zeiss) objective, the volume fractions of parenchymal tissue, V_V(par/lung), and the volume fractions of alveoli within parenchymal tissue, V_V(alv/par), were determined. Parenchyma was defined as the gas exchange region, excluding large blood vessels and bronchi. Alveoli were defined as alveolar lumen and septae, excluding respiratory bronchioles and alveolar ducts. This made necessary a decision as to whether a test point hits the lumen of an alveolus or an alveolar duct. In these cases, a straight, imaginary line was drawn between the free edges of the alveolar walls. This is an approximation of consequence for the estimation of alveolar volume but of no consequence for the estimation of alveolar number.

The number of alveoli was then determined by the estimation of the Euler number (χ_3) (18) of the network of alveolar openings. The Euler number or Euler–Poincaré characteristic, named after the Swiss mathematician Leonhard Euler (1707–1783), can be defined as an integer-valued measure for the connectivity of an object. The Euler number of a two-dimensional network in a three-dimensional space depends on the number of connections in the network (here: the number of alveolar openings). Using the two adjacent sections as physical disectors for counting in both directions, i.e., using each single section once as a sampling section and once as a look-up section, new alveolar openings appearing between the two sections were counted (*see* Figure 1). These counting events were ascertained as bridges (B). The very rare occurrence of new, isolated alveolar edges without connections to previously visible septae was ascertained as islands (I) (*see* References [12, 18] for further details). New closed alveolar profiles that appeared were not counted because they do not represent the two-dimensional network of alveolar openings. Using the disectors to count in both ways, the contribution from the disector counts to the total Euler number is then determined as

$$\Delta\chi_3 := \left(\sum \mathrm{I} - \sum \mathrm{B}\right)/2 \tag{1}$$

where := signifies that the expression is an estimator, not that of an identity, and the subscript 3 indicates that the two-dimensional network of alveolar openings is analyzed in a three-dimensional space (the disector is a three-dimensional stereologic probe).

The numerical density of alveoli in the parenchyma is

$$N_V(\mathrm{alv/par}) := -\sum \Delta\chi_3(\mathrm{alv})/\sum \nu(\mathrm{par}) \tag{2}$$

The total Euler number is estimated as

$$\chi_3 := \left[\sum \Delta\chi_3/\sum \nu(\mathrm{dis}) \cdot \mathrm{V(ref)}\right] \tag{3}$$

where the term in brackets is the density (number-to-volume ratio) of alveolar openings in the lung parenchyma. The total volume of the disectors used for counting (Σ v(dis)) is determined as

$$\sum \nu(\mathrm{dis}) = \mathrm{h} \cdot \mathrm{n} \cdot \mathrm{a(frame)} \tag{4}$$

with h being the height of the disector (in this case the section thickness because adjacent sections are used) and n being the number of disectors with a frame area a(frame).

The reference space [V(ref)] was defined as the total volume of parenchyma [V(par)] and was estimated as

$$\mathrm{V(par)} := V_V(\mathrm{par/lung}) \cdot \mathrm{V(lung)} \tag{5}$$

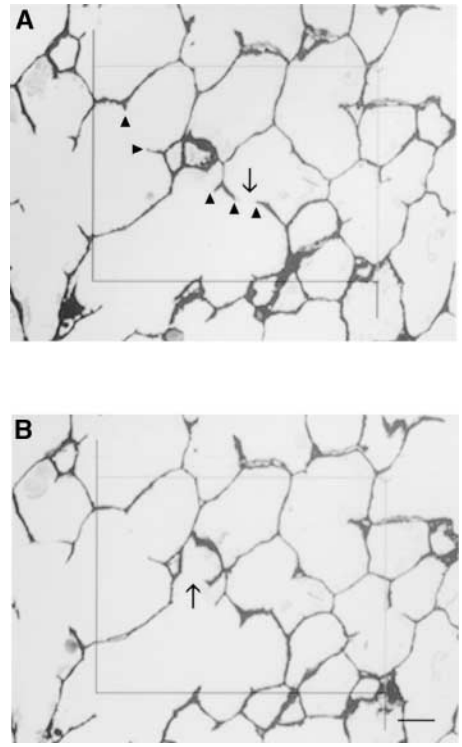

Figure 1. Physical disector pair at the light microscopical level demonstrating the counting method. The two-dimensional network of alveolar openings is represented by the free edges of the alveolar septae (indicated by *arrowheads* in *A*). Using two adjacent sections with a thickness of 9 μm, corresponding areas (shown in *A* and *B*) are compared using the unbiased counting and sampling frame. Only events completely inside the frame or intersecting the two inclusion edges (*thin*) are considered, and any event intersecting the exclusion lines (*thick*) is not sampled (8). Free ends of alveolar septae that become connected in the adjacent section are ascertained as bridges (*arrows*). Whenever there is a new bridge appearing in a disector, there is a new alveolar opening that is counted. The appearance of new, isolated alveolar opening edges, ascertained as islands, is a very rare event (not shown). The bottom of an alveolus does not contribute to the network of alveolar openings, i.e., new closed alveolar profiles appearing in a disector are not counted because no new alveolar opening becomes visible (not shown). Counting is performed in both directions, i.e., using each single section (*A* or *B*) once as sampling section (for counting) and once as look-up section (for comparison). *Scale bar* = 50 μm.

Finally, the estimator of the total number of alveoli in a lung is

$$\mathrm{N(alv)} := -\chi_3 \tag{6}$$

The mean size of an individual alveolus [$\bar{v}_N$(alv)] was then estimated indirectly by dividing the total alveolar volume by the alveolar number

$$\bar{v}_N(\mathrm{alv}) := \frac{\mathrm{V(alv)}}{\mathrm{N(alv)}} \tag{7}$$

with V(alv) being estimated as

$$\mathrm{V(alv)} := V_V(\mathrm{alv/par}) \cdot \mathrm{V(par)} \tag{8}$$

To evaluate the contribution of the stereologic estimation procedure to the total observed variation (CV_{obs}), the coefficient of error (CE) of the stereologic procedure was estimated (*see* online supplement).

Additional details on the method, including a table with descriptions of all parameters as well as an example using actual numbers from one lung in the equations to illustrate how to calculate all stereologic estimators in practice, are given in the online supplement.

RESULTS

Stereology

The stereologic results for the six lungs are summarized in Table 1 (for individual data, *see also* Table E1 in the online supplement). The mean number of alveoli in the six single lungs investigated was 240 million, with a large interindividual variation (CV_{obs} = 37%), which was clearly reflected in the total lung volume as well (CV_{obs} = 34%). The architecture, the volume fractions of parenchymal tissue (92%) and alveoli within the lung (64%), was remarkably constant (CV_{obs} = 3%), as was the mean alveolar volume of 4.2×10^6 μm^3 with a CV_{obs} of 10%. As expected, both total lung volume and total number of alveoli showed a clear sexual dimorphism: The CV_{obs} within sexes (16% for total lung volume and 23% for total number of alveoli) represented less than half of the total observed variance. Because three right and three left single lungs were analyzed, the mean value for N(alv) can be doubled to get an unbiased estimate of 480 million alveoli for the number of alveoli in a complete pair of lungs. In a larger population where males and females are represented equally, the mean value will probably be slightly higher, i.e., a little above 500 million alveoli per double lung. One cubic millimeter lung parenchyma would then contain approximately 170 alveoli. In the six lungs investigated, alveolar number was closely related to total lung volume, with larger lungs having considerably more alveoli (Figure 2A). In contrast, the mean size of a single alveolus showed no correlation with total lung volume (Figure 2B).

In stereologic studies of a group of individuals, the total observed variation of an estimate (CV_{obs}) is influenced by the biological variation between individuals (which is fixed for a given population) and by the variation due to the methodology used (CE; which can be decreased by increasing the amount of sampling within one individual). The central question if one has "counted enough" (achieved a sufficient precision) in a stereologic study can therefore only be answered if these factors are known. It is considered sufficient if the CE of the stereologic estimate is not the major factor contributing to the CV_{obs}. Of the CV_{obs} of 23% within sexes, the CE of 8.7% for the Euler number estimation constitutes only a small fraction of 7.4%, indicating an almost too good precision of the stereologic estimate. The remaining variation can mostly be attributed to a rather large biological variation among the individual lungs. Moreover, after adjusting the sampling and counting design at the beginning of the study, which takes approximately 3 hours at the microscope, a complete stereologic analysis of one lung takes only around 7 hours. The efficiency of a stereologic method can be expressed as precision per time or per unit cost. Thus, the method is very efficient (provides a high precision within a short time).

TABLE 1. SUMMARIZED STEREOLOGIC RESULTS

Parameter	Mean	SD	CV_{obs} (%)	Range
V(lung), cm^3	1,534	521	34	1,031–2,372
V_V(par/lung), %	92	3	3	88–96
V_V(alv/par), %	70	3	4	66–75
V_V(alv/lung), %	64	2	3	62–66
N(alv), 10^6	240	89	37	137–395
$\bar{v}_N$(alv), 10^6 μm^3	4.2	0.4	10	3.3–4.8

Definition of abbreviations: CV_{obs} = total observed variation; V = volume; V_V = volume fraction; N = number; $\bar{v}_N$ = number-weighted mean volume, par = parenchyma, alv = alveoli.

Summarized stereologic data for six single human lungs (for individual data, *see* Table E1 in the online supplement).

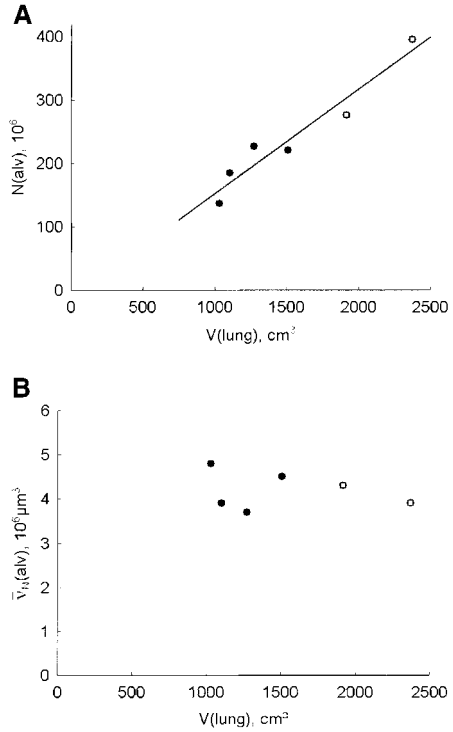

Figure 2. Human single lung alveolar number versus lung volume (*A*) and mean alveolar volume versus lung volume (*B*). Females are indicated by *black dots*, males are indicated by *white dots*. Whereas alveolar number is closely related to total lung volume with larger lungs having more alveoli (*A*) (r = 0.964; p = 0.002; regression line origin at y = −12.5), alveolar size remains rather constant showing no correlation with total lung volume (*B*) (r = −0.239; p = 0.648).

DISCUSSION

The aim of the present study was to establish a new design-based stereologic approach for the direct and unbiased estimation of alveolar number (*see* Reference 12) and to provide reference data for the human lung. The six single lungs examined in the present study were fixed by airway instillation to ensure rapid and uniform fixation of the whole organ for subsequent stereologic investigation. Samples taken from these lungs according to systematic, uniformly random sampling procedures by definition represent the whole organ equally well, thus avoiding any sampling bias that may be seen in biopsy specimens (*see* Reference 15). In contrast to adult human lung tissue obtained from surgical specimens removed for malignant tumor, which is the "normal" tissue usually available for morphologic analysis, the material investigated in the present study can be regarded to represent normal healthy adult human lungs (*see also* References 14 and 19).

Organ volume is the basic reference space to which all stereologic estimates obtained as densities by microscopic techniques are usually related to. In respiratory biology, the volume of the lung is therefore a critical parameter in any stereologic study, which should be measured as close to the subsequent sampling and embedding steps as possible (20). Especially for larger lungs, lung volume measurement by a method based on the Cavalieri principle (21) is preferable to the fluid displacement method because it allows an estimate in a state free of residual tissue elasticity (20). For dog lungs, volumes obtained by fluid displacement even after pressure release were found to be 14% higher than by the Cavalieri method (20). This corresponds well

with our data in a series of human lungs where fluid displacement yielded values 16% higher than those obtained by the Cavalieri method (Voigt and Ochs, unpublished results).

The stereologic method for the estimation of alveolar number that is presented here is free of assumptions about the shape, the size, or the spatial orientation or distribution of alveoli and therefore fulfils the criteria for design-based or, because unbiasedness is a built-in property of design-based methods, unbiased stereology (22). Although the disector method is well known to have revolutionized the scientific basis of counting and sizing of discrete particles from sections (22, 23), it has been used less frequently for connectivity estimation. For arbitrary networks, the disector provides an unbiased estimate of the so-called Euler number (18, 24). Alveolar openings are ordinarily the real problem when estimating alveolar number because they make the particle boundary incomplete and thus impede the distinction between alveolar and nonalveolar parenchyma, especially on thin histologic sections. Because alveoli are not discrete particles due to their openings into alveolar ducts or respiratory bronchioles and thus are not isolated, countable structures in any ordinary sense, a rigorous topologic definition is necessary for counting. The basic idea behind the method described and applied here is to concentrate on the alveolar openings and to use their appearance or disappearance in a physical disector as counting events. This reduces the examination of the complex parenchymal architecture seen under the light microscope to the analysis of a two-dimensional network of alveolar openings in a three-dimensional space. In practice, one simply has to focus on the edges of alveolar walls as seen in the histologic sections. If necessary, specific staining of elastic fibers, which are known to be preferentially localized at the edges of alveolar walls (entrance rings), will be helpful to visualize the two-dimensional network of alveolar openings.

Depending on the overall sampling strategy adopted, total alveolar number and alveolar size can be estimated using either a fractionator design (i.e., multiplying total counts with the sampling fraction, *see* Reference 12) or a combination of the disector and Cavalieri method (i.e., multiplying a density with a reference volume). In the present study, the sampling design used for the human lung material available to us only allowed a disector–Cavalieri approach. In case of a disector–Cavalieri combination, technical bias may be introduced due to tissue shrinkage during processing, embedding, and sectioning. However, the use of glycol methacrylate as embedding medium as well as results from control experiments (*see* online supplement) make it unlikely that significant shrinkage occurred in our material. In contrast to the disector–Cavalieri approach, a fractionator design would allow estimation of total numbers without the need to know the volume of the reference space, thus being independent of shrinkage (13). Sometimes however, especially in human lung studies, a fractionator design may not be feasible, but total lung volume estimation by the Cavalieri method might be possible using computerized tomography or magnetic resonance imaging scans.

Using various methodologic approaches, the number of alveoli in the human lung has been estimated several times. An estimate of alveolar number in the human lung based on a geometric model was provided more than 40 years ago by Weibel and Gomez (25). Their approach was based on the relationship (25, 26)

$$N_V = \frac{N_A^{(3/2)} \cdot K}{\beta \cdot V_V^{(1/2)}} \tag{9}$$

where N_V is the number of alveoli in a unit volume of tissue, N_A is the number of alveolar profiles in a unit section area, K is the size distribution coefficient for alveoli, β is the shape coefficient for alveoli, and V_V is the volume density of alveoli. In a series of five single human lungs from three males and two females, aged 8 to 74 years, a very constant number of approximately 300 million alveoli were found. This approach has since been used by others, mainly confirming the data obtained by Weibel and Gomez. Dunnill estimated a mean number of 286 million alveoli in a human lung of a 55-year-old female (27). In 32 human lungs aged 19 to 85 years, Angus and Thurlbeck estimated a mean of 375 million alveoli (28). However, this method is not unbiased because it depends on many assumptions regarding particle shape, size distribution, and orientation, and should therefore no longer be used (7). An additional practical problem with this method is the difficulty in counting alveolar profiles in single thin sections (29). Instead, an assumption-free estimate of the total number of objects in space (a dimensionless quantity) can only be obtained with three-dimensional probes like the disector (8). If a disector is used for the estimation of particle size without knowing the section thickness, it is referred to as a selector (30). An indirect approach to determine alveolar number based on the estimation of alveolar size using the disector/selector principle has been applied in rodents (31). A first estimate of the number of alveoli in the human lung based on disector counts has been given by Mercer and coworkers (32). Using resected lobes from three patients, one male and two females, aged 19 to 43 years, the authors estimated a mean number of 486 million alveoli, a value that corresponds well with our present data, considering the assumptions on which it is based (nonuniform cluster sampling and incomplete counting rules were used).

Counting alveolar openings with physical disectors allows the direct estimation of alveolar number, and any problems related to difficulties in defining alveolar boundaries in single histologic sections are avoided. By dividing total alveolar volume by alveolar number, the mean size of an individual alveolus can then be estimated indirectly. In contrast to earlier data (25), our results, based on six single human donor lungs, yielded large variations in alveolar number (CV = 37%; CV of lung volume was 34%) and a rather constant alveolar size (CV = 10%). A large variation in the number of alveoli in normal human lungs has already been pointed out (28). Furthermore, the present results show a close relationship between lung volume and alveolar number in the lungs investigated. Thus, from our data we may conclude that, in humans, larger lungs are built by increasing the number of alveoli and not by increasing their size. A similar relationship between the number and size of cells in the alveolar region seems to exist between various mammalian species (33).

The easy recognition of counting events and the high efficiency should allow for a widespread application of the stereologic method employed here. It can be used in all studies where alveolar development and remodeling need to be quantitated. Possible future applications of the method include the estimation of alveolar number during normal postnatal lung development as well as in cases of abnormal lung maturation, e.g., due to hypoxia and hormonal influences (34) or due to inflammatory processes in "new" bronchopulmonary dysplasia (35–37). Pathologic alterations of alveolar number in the adult lung, e.g., in emphysema, might also be assessed by this approach. The method may also be helpful to differentiate between simple enlargement of terminal airspaces (as revealed by an increase in mean linear intercept length) and true destructive emphysema characterized by loss of alveoli and, therefore, loss of gas exchange surface in animal models of emphysema. The method might also be applied to answer the question whether compensatory lung growth after pneumonectomy occurs by formation of new alveoli or by enlargement of existing alveoli. The potential significance of the method in terms of diffusing capacity and $\dot{V}/\dot{Q}$ matching remains to be shown in comparative morphologic studies.

The method for counting alveoli presented here is based on the fact that alveoli have only one opening (into an alveolar duct or a respiratory bronchiole). A clear distinction between the "true" opening of an alveolus and interalveolar openings

(pores of Kohn) is therefore necessary to guarantee an unambiguous recognition of counting events. Although this should not be difficult in normal lungs, this might become a practical problem in cases of severe emphysema. Erroneous counting of enlarged pores of Kohn as alveolar openings would lead to an overestimation of alveolar number. However, experience in lung morphology and the use of adequate staining techniques for the demonstration of elastic fibers known to be concentrated in alveolar entrance rings (32) should allow the identification of the "true" alveolar opening in almost all cases.

In conclusion, a design-based stereologic method for the unbiased estimation of alveolar number and size has been applied to the human lung. It is very efficient and easy to apply in practice. Using this method, a mean number of 480 million alveoli with a mean size of 4.2×10^6 μm^3 (roughly a diameter of 200 μm) was found in six human lungs. Alveolar number was closely related to total lung volume whereas alveolar size was not. The move away from stereologic methods based on assumptions on the shape, size, or orientation of objects to design-based stereologic methods that are free of any of these assumptions has been considered a major scientific advance (22). This has been appreciated especially in the neurosciences and in nephrology where established journals began to require these state-of-the-art techniques for counting of particles (23, 38–40). Given the long tradition in lung stereology (*see* References [3, 4]), we believe that the time has come in respiratory biology as well to make clear statements regarding the methods that are expected to report quantitative structural data.

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